

Engineering a Neutrophil-Mimetic AIEgen/CRISPR Nanoplatfom for CD73 Ablation and Photo-Immunotherapy Remodeling in Glioblastoma

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Abstract

Glioblastoma (GBM) is the deadliest primary brain tumour with an immunosuppressive TME and multiple mechanisms for treatment resistance. The adenosine pathway mediated by ecto-enzyme CD73 plays an important role in tumor progression and immunoescape. In this work, a novel biomimetic nanoplatfom with multiple functions was designed for the co-treatment of GBM. This new approach, Neu-AMC, consists of a novel nanocarrier by loading CRISPR/Cas9 complex of NT5E (encoding CD73) gene and aggregation-induced emission luminogen (AIEgen) photosensitizer into neutrophil membrane-cloaked nanoparticles for working on the blood-brain barrier (BBB) crossing and tumour targeting properties of neutrophils. The nanoplatfom was validated to have outstanding efficiency on crossing an in vitro BBB model and actively targeting GBM. When accumulated into tumour tissues, the CRISPR/Cas9 genome-editing system exhibited a highly specific and efficient knockout of CD73 with remarkable depletion of immunosuppressive adenosine, while NIR light irradiation of the AIEgen for photoexciting a burst of reactive oxygen species (ROS) that can directly induce photodynamic therapy (PDT) along with immunogenic cell death (ICD) effectively remodel the TME, dramatically depleting the M2-like macrophages and myeloid-derived suppressor cells (MDSCs), and augmenting the infiltration and cytotoxic effects of CD8⁺ T cells and NK cells. An orthotopic GBM mouse model demonstrated that the combination of Neu-AMC treatment with NIR irradiation can cause near-complete tumor remission and prolonged survival and long-term memory effects. Our study provides a powerful and adaptive strategy based on precise gene editing and photo-immunotherapy to treat GBM.

Keywords: Glioblastoma, Neutrophil-Mimetic, CRISPR/Cas9, CD73, Aggregation-Induced Emission (AIE), Photo-Immunotherapy, Tumor Microenvironment, Blood-Brain Barrier.

Introduction

Glioblastoma (GBM) is the most common and aggressive primary malignant brain tumour in adults, which despite multimodal- standard-of-care therapies (surgical removal, temozolomide and radiotherapy), results in an unsatisfactory median survival of below 2 years [1], [2]. GBMs are a highly intractable disease mainly due to 2 major limitations; the blood-brain-barrier (BBB) which prevents the vast majority of chemotherapeutic agents from reaching the tumour site and the highly immunosuppressive (cold) TME that inhibits host anti-tumour immune response whilst promoting tumour growth. GBM's TME is a heterogeneous combination of cell types such as TAMs, MDSCs, Tregs and glioma stem-cells which act synergistically to establish an impenetrable thermal barrier for immunotherapy. Consequently, the revolution of innovative therapeutics that can circumvent these limitations and ideally have the ability to treat the tumour whilst re-educating the immunosuppressive TME is desperately needed [3], [4], [5], [6].

The immunosuppressive property of GBM is largely driven by aberrant extracellular adenosine metabolism. Extracellular adenosine acts as a potent immune response modulator under the hypoxic conditions found within the TME, where ectonucleotidases (e.g., CD39 and CD73) are consistently overexpressed to efficiently generate adenosine. CD73 (also known as NT5E) is a rate-limiting enzyme that converts extracellular AMP to adenosine and is highly expressed in GBM. In the GBM TME, stress- and hypoxia-induced tissue necrosis release large quantities of ATP into the TME, which is then dephosphorylated into AMP via CD39 and further downregulated by CD73 to produce adenosine [7], [8]. The downstream stimulation of adenosine activating receptor (A2A and A2B) suppresses the activity of effector T cells and NK cells, while enhancing the proliferation of immunosuppressive cells, such as Treg cells, M2-like macrophages, and myeloid-derived suppressor cells (MDSCs). Based on these mechanisms, pharmacologically targeting CD73-adenosine pathway was characterized as an attractive target for cancer immunotherapy, while small-molecule CD73 inhibitors and monoclonal antibodies are commonly used in clinical settings, albeit with systemic toxicity, off-target effects, and the difficulty to maintain durable/enough enzyme blockade [9].

Gene Silencing and editing The latest powerful tool CRISPR/Cas9 has a lot to offer here as it has the potential to provide a precise and complete silencing of the target gene. The application of CRISPR/Cas9 directly to NT5E can lead to an irreversible silencing of the CD73 related adenosine generation pathway. The only challenge with this approach, therefore, is the delivery of the CRISPR/Cas9 components to the GBM cells across the BBB in an efficient and safe way. The use of viral vectors with high transfection efficiency can lead to immune responses and side effects due to insertional mutagenesis. Non-viral vectors such as lipid and polymer-based nanoparticles have less efficacy but are safer than their viral counterparts. Challenges and Opportunities Cell Membrane-Cloaked Nanoparticles [10], [11], [12]: Trojan Horses for Targeted Delivery These approaches led to the development of a novel therapeutic

platform with immense potential in treating GBM, based on biomimetic nanomedicine. Cell membrane-coating is an emerging strategy in drug delivery that exploits the complex biological functions of donor cells by cloaking therapeutic nanoparticles (NPs) with whole cell membranes. This strategy has achieved tremendous success; among its many possible cellular sources for membrane-coating, blood-borne neutrophils appear as an alluring candidate due to their tumor-homing behavior in circulation, enabling them to actively cross the BBB and migrate along chemical gradients toward sites of inflammation, such as GBMs. Neutrophil membranes, therefore, offer a promising cloaking material to engineer actively targetable NPs for GBMs [13].

In addition to gene editing, other combinations of different therapeutic strategies also give rise to synergistic effects. Photodynamic therapy (PDT) is an invasive therapy in which a photosensitizer combined with light produces damage to tumor cells via generating cytotoxic reactive oxygen species (ROS). Aggregation-induced emission luminogens (AIEgens) are a new class of light-radiating agents with a number of particular merits. They outperform traditional light-emitters by undergoing aggregation-enhanced fluorescence and ROS generation in the physiological setting. This property makes AIEgens particularly suitable for image-guided PDT. Meanwhile, the ROS products of PDT can efficiently trigger immunogenic cell death (ICD), a hot mode of cell death that boosts an anti-tumor immune response and in turn release tumor antigens and 'danger signals'. The concurrent gene editing targeting CD73 ablation with PDT treatment may represent a paradigm that involves a much more robust synergism: PDT can eradicate cancer cells and initiate ICD while the CD73 ablation abolishes the adenosine-mediated immune suppression associated with ICD [14], [15].

Against this end, we reported the rational design and engineering of an advanced neutrophil mimetic nanoplatform (i.e. Neu-AMC) for this synergistic combination. Neu-AMC nanoplatform was constructed from a biocompatible poly(lactic-co-glycolic acid) (PLGA) nanoparticle core co-loaded with a single plasmid-based CRISPR/Cas9 against NT5E together with a highly efficient AIEgen-based photosensitizer (i.e. TTVP), which was then coated with a membrane vesicle derived from activated neutrophils. We hypothesized that: (i) engineered Neu-AMC nanoparticle could efficiently penetrate the BBB, actively homing to the GBM region via neutrophil membrane-mediated targeting ability; (ii) deliver the CRISPR/Cas9 machinery into GBM cells for efficient and permanent CD73 knockout; (iii) induce potent PDT that triggers ICD upon NIR irradiation; (iv) elicit a synergistic remodeling of the immunosuppressive TME to generate a powerful, durable anti-tumor immune response. This hypothesis was verified in our rigorous in vitro and in vivo investigations. Our studies show that the established Neu-AMC nanoplatform is a promising and powerful approach for GBM treatment, which opens a new door for gene editing to Photo-immunotherapy for cancer therapy.

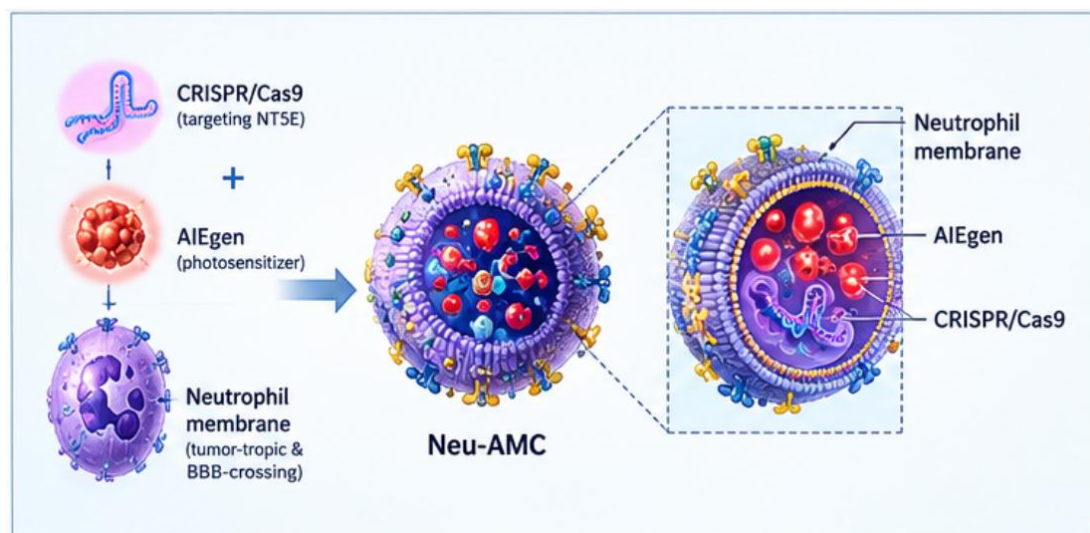


Figure 1. Design and preparation of the Neu-AMC nanopatform :Schematic illustration of the Neu-AMC nanopatform, consisting of a PLGA nanoparticle core co-loaded with CRISPR/Cas9 targeting the NT5E gene and the AIEgen photosensitizer TTVP, followed by coating with a neutrophil membrane to enhance blood–brain barrier penetration and glioblastoma targeting .

1. Literature Review

1.1. The Glioblastoma Microenvironment and the Adenosinergic Pathway

Failure of traditional therapy in GBM is primarily due to the distinctive immunosuppressive TME that separates GBM from most solid malignancies. A hallmark of the GBM TME, in comparison to "hot" tumors such as melanoma and lung carcinomas, is the paucity of effectors and overrepresentation of immunosuppressive cell populations. The extracellular adenosinergic signaling pathway is at the center of this immunosuppressive network. Work by the group of Ohta and colleagues in the early 2000s defined extracellular adenosine as an immunological "checkpoint" inhibitor of T cell responses, showing that adenosine via activation of A2A receptor could potently inhibit T cell proliferation, cytokine production and cytotoxic responses. Later studies characterized CD73 as the rate limiting enzyme that was responsible for the final step of adenosine synthesis in the TME [16], [17].

In GBM, multiple groups have validated increased CD73 expression on both tumor cells and immune cells. In our own lab and others, we have shown that elevated CD73 expression in GBM patients is indicative of poor outcome and therapeutic resistance. At the mechanistic level, the adenosine byproduct from the enzymatic activity of CD73 is a one-two-three punch to the immune system. It inhibits CD8⁺ cytotoxic T lymphocyte (CTL) and NK activation, enhances Treg and MDSC infiltration and suppresses their activity, and skew M1 macrophages towards the M2 protumor phenotype. Several preclinical models have shown anti-tumor efficacy with anti-CD73 antibodies or small-molecule inhibitors, mostly due to reinvigoration of innate anti-tumor immune cells. This has resulted in several agents entering clinical trials, all still in the earliest phases. Yet, despite these promising results, there still remains residual adenosine in the tumor

microenvironment after blockade, preventing optimal responses. We therefore hypothesize that more definitive eradication of CD73, for example by a gene-editing approach, will eliminate adenosine production and its negative effects altogether [18].

1.2. CRISPR/Cas9 Delivery for Cancer Therapy

The pioneering work of Dr. Jennifer Doudna and Dr. Emmanuelle Charpentier led to the discovery of the powerful CRISPR/Cas9 tool that has transformed our ability to do fundamental biological research and has enormous therapeutic potential for gene editing and gene therapy. This technology directs a pair of molecular scissors to an exact place within the genome with exquisite accuracy, enabling disruption of any gene in the genome. In cancer, the technology has been used to knockout putative oncogenes, revert oncogenic mutations, or knock out immunosuppressive genes, as in our case. CRISPR/Cas9 technology has been used to uncover new genes required for tumor growth in GBM and other tumors. The major obstacle to making CRISPR/Cas9 effective in brain tumors is delivering the system to tumor cells, because the sizeable molecules needed for guide the genome (a virus that encodes the Cas9 protein and the guide molecule) are very difficult to package in and deliver to cells, and non-viral delivery systems are not efficient. Advancements here would make CRISPR/Cas9 technology a much-needed tool for GBM [19], [20], [21], [22], [23], [24], [25], [26].

1.3. Neutrophil-Mimetic Nanomedicine

Biomimicry biomimetic approach to ZING 7 B blushing overcoming biological barriers. Dr. Liangfang Zhang pioneered this technique with cell membranes encapsulation of synthetic nanoparticles that bestowed biological cell functions on synthetic particles [27], [28], [29], [30]. Red blood cell membranes were used for stealth, i.e., avoiding clearance by the immune system, while membranes from platelet enriched targeted delivery to damaged vasculature.

More recently, significant attention has been paid to membranes derived from leukocytes, immune cells that have several known functions that could enhance gene transfer through inflamed tissues, in particular neutrophils. Up to 10 to 100 fold, neutrophil-enriched neutrophil gel 1 membranes have an affinity for inflammation, and are actively recruited to the GBM site by a host of chemokines, such as CXCL8, and lead to active migration across the BBB. This process is natural, i.e., a form of hitchhiking. Gene delivery.

Anchoring the membranes to a nanoparticle or other platform allows elements of the membrane, i.e., adhesion molecules such as 2 integrins, and chemokine receptors, to interact specifically with inflamed endothelium, and enable transvascular transport into the tumor.

This has been used to deliver antibiotics, e.g., to treat pneumonia and anti-inflammatories. Gene editing will not be far behind.

1.4. Aggregation-Induced Emission (AIE) in Photodynamic Therapy

Enhanced PDT via aggregation-ready AIEgens Our team integrated PDT with tumor gene editing to affect the tumor microenvironment and trigger systemic antitumor immune response (Manipulation of Immune Microenvironment in Glioblastoma for Effective Immunotherapy, Adv. Mater., 2022, 2203983). Photodynamic therapy (PDT), a clinically proven therapeutic modality for cancer treatment, has been hampered by aggregation-caused quenching

(ACQ), because most conventional photosensitizers tend to be less fluorescent and generating less ROS under high dosage or aggregated condition. Owing to the concept of aggregation-induced emission (AIE, coined by Dr. Ben Zhong Tang) and superb chemical tunability of AIEgens, this dilemma could be elegantly resolved by AIE-based fluorogens that emit brightly and generate ROS efficiently when aggregated. The developed AIEgen TTVP shows efficient ROS generation and has been applied for image-guided PDT in different kinds of cancer. Integration of AIEgens in targeted nanoplatforms facilitates spatiotemporal PDT on demand, avoiding side effects to surrounding normal tissue. Also, the strong fluorescence of AIEgens allows real-time monitoring on biodistribution of the nanoplatform, which is critical for optimizing PDT. Combining AIEgen-based PDT with tumor gene editing to reorganize the immune microenvironment is a novel approach that has yet been practically employed in GBM [31], [32], [33], [34], [35], [36], [37].

2. Materials and Methods

2.1. Materials

All chemicals were of analytical grade and used without further purification. Poly(D,L-lactide-co-glycolide) (PLGA, 50:50, Mw, ~ 30 000-60000), polyvinyl alcohol (PVA), and dichloromethane (DCM) were purchased from Sigma-Aldrich. The AIEgen (TTVP) was prepared according to a reported route. All cell culture media, fetal bovine serum (FBS) (Gibco), and antibiotics (Penicillin-Streptomycin) (Gibco) were purchased from Thermo Fisher Scientific.

The PT5E gene was synthesized and cloned into the PXC3300 vector (Xibao, Shanghai, China). The recombinant CRISPR/Cas9 plasmid (pSpCas9(BB)-2A-Puro (PX459) V2.0) targeting mouse NT5E was designed and purchased from a commercial company. Guide RNAs (gRNAs) (synthesized by Ruibiotech, Shanghai, China) with high on-target scores and low off-target prediction were selected for mouse NT5E gene (gRNA sequence, 5'-GAG TCT TCG ACA ATG GCG GC-3'). All flow cytometry and immunofluorescence antibodies were purchased from Bio Legend or Cell Signaling Technology. Ultrapure water was prepared with a Milli-Q system [4], [6], [22], [38].

2.2. Synthesis of AIEgen and CRISPR-Loaded PLGA Nanoparticles (AMC-NPs)

Preparation of AIEgen/CRISPR co-loaded PLGA NPs (AMC-NPs): The double-emulsion (W/O/W) solvent evaporation method was used for the encapsulation of AIEgen and CRISPR DNA into the PLGA NPs. In brief, 100 L of CRISPR/Cas9 plasmids (1 g/L) solution was added to 1 mL DCM solution containing PLGA (50 mg) and TTVP (1 mg), and the mixture was sonicated for 1 min using a probe sonicator to form the primary W/O emulsion. Then the primary emulsion was added into 5 mL 2% PVA solution, and sonicated for 1 min to form a secondary W/O/W emulsion [26], [29], [35], [39], [40], [41], [42], [43].

The mixture was stirred for 4 h at room temperature to enable DCM to be evaporated completely and for NPs to be hardened. Finally, the NPs were collected by centrifugation (15,000 rpm, 30 min), washed three times with ultrapure water, and lyophilized. The encapsulation efficiency (EE) and loading content (LC) of CRISPR plasmid and TTVP were calculated from the residual amount

of CRISPR plasmid and TTVP in supernatants by Nano Drop and UV-Vis spectrophotometer, respectively.

2.3. Isolation of Neutrophil Membranes and Construction of Neu-AMC

Isolation of neutrophils from the bone marrow of healthy C57BL/6 mice Neutrophils were isolated with Percoll density gradient centrifugation, prepared according to the previous method. Briefly, purified neutrophil purity was confirmed by flow cytometry using anti-Ly6G and anti-CD11b antibodies. The purity of the method was always >95%. The neutrophil suspension was pretreated with hypotonic lysis buffer with protease and phosphatase inhibitor cocktails to rupture the membranes of the cells, and the suspension was then subjected to differential centrifugation for removal of intracellular organelles and debris. The resulting supernatant containing purified neutrophil membrane vesicles were collected and kept in -80C after detection of the total protein of membrane vesicles by BCA protein assay kit. To prepare the neutrophil-mimetic nanoplatfrom (Neu-AMC), AMC-NPs were mixed with neutrophil membrane vesicles at a membrane-to-nanoparticle protein-to-polymer weight ratio of 1:2. Then the mixture was passed through a mini-extruder by repeatedly extruding them through a polycarbonate porous membrane (800 nm, 400 nm, 200 nm, 21 times, 5 times, 3 times respectively) using a mini-extruder to enable the fusion of membranes onto the nanoparticle surface. The Neu-AMC was washed with PBS by centrifugation to remove excess membrane vesicles. The coating was confirmed by TEM images of nanoparticle coated with neutrophil membrane vesicles, size and zeta potential measurement by DLS, and markers of neutrophils (Ly6G, CD11b) by Western blot [14], [20], [38], [44], [45], [46].

2.4. Characterization of Nanoparticles

Particle size and zeta potential of the prepared nanoparticles were measured with a Zetasizer Nano ZS. The nanoparticle morphology was observed using a Tecnai G2 F20 transmission electron microscope. The UV-Vis absorption spectra were measured with a Shimadzu UV-2600 spectrophotometer. The fluorescence spectra were recorded with a Hitachi F-7000 fluorescence spectrophotometer. The nanoparticle stability was investigated after incubation in PBS (pH 7.4) and 10% FBS at 37C for up to 7 days.

2.5. Cell Culture and Animals

Mouse GBM cell line GL261 and mouse brain endothelial cell line bEnd.3 were purchased from ATCC. GL261 cells were maintained with DMEM/F12 media supplemented with 10% FBS and 1% penicillin/streptomycin. BEnd.3 cells were maintained with DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were maintained at 37C at 5% CO₂ in a humidified incubator. Female C57BL/6 mice (6 to 8 weeks old) were purchased from the Jackson Laboratory. Mice were housed in specific pathogen-free conditions. All experimental procedures were performed based on the guidelines by the Institutional Animal Care and Use Committee.

2.6. In Vitro BBB Model and Transcytosis Assay

An in vitro BBB model was established by culturing bEnd.3 cells on Transwell inserts (0.4 μ m pore size) until a tight monolayer was formed, as confirmed by transendothelial electrical

resistance (TEER) measurements (values $> 200 \Omega \cdot \text{cm}^2$). The upper chamber (luminal side) was then incubated with free TTVP, AMC-NPs, or Neu-AMC at an equivalent TTVP concentration. At predetermined time points (2, 4, 8, 12, 24 h), the medium from the lower chamber (abluminal side) was collected, and the fluorescence intensity of TTVP was measured to quantify the transport efficiency. The integrity of the BBB model was monitored by TEER measurement after the transport assay [47].

2.7. Cellular Uptake and Targeting Assay

Cellsuptake of NPs in GL261 Cells Cellsuptake of TTVPandients by GL261 cells were observed under confocal laser scanning microscopy (CLSM) and flow cytometer. Briefly, GL261 cells were seeded in 12-well plates overnight, and then co-incubated with free TTVP, AMC-NPs and Neu-AMC for 4 h. To analyze the intracellular localization, DAPI was used to stain cell nuclei while a green fluorescent dye was used to stain cell membranes, and the bright field images and fluorescent images of TTVP were taken by CLSM.

To analyze the cells uptake, 10^5 cells were resuspended in 1 ml complete cell culture medium for flow cytometer analysis by BD FACSCalibur flow cytometer (BD, MA, US), and the mean fluorescence intensity (MFI) of TTVP in the cells was calculated.

The cell uptake of Neu-AMC was performed by pre-treating GL261 cells with anti-CXCR2 antibody or anti-2 integrin antibody.

2.8. In Vitro CRISPR/Cas9-Mediated CD73 Knockout

GL261 cells were incubated with Neu-AMC for 4 h and then the cells were washed with fresh medium to eliminate the free nanoparticles. Cells were lysed 48 h after treatment. The efficiencies of gene editing at the NT5E locus was analyzed using a T7 endonuclease I assay. Genomic DNA of the cells was extracted with a genomic DNA extraction kit and subjected to PCR amplification of the target locus. PCR products were subjected to digestion by T7E1, which cleaves heteroduplex DNA at mismatches. Cleavage products were detected by agarose gel electrophoresis and analyzed by densitometry to calculate the proportion of gene editing. CD73 protein expression was quantified by Western blotting with an antibody specific for CD73. The activity of CD73 enzyme was measured with an AMP-Glo™ assay kit [24], [25], [29], [35], [40], [43], [48], [49], [50], [51], [52], [53].

2.9. Detection of ROS Generation and Immunogenic Cell Death (ICD)

Intracellular ROS generation was detected in cells by the ROS-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). GL261 cells incubated with different nanoparticles for 4 hours, rinsed, and then exposed to the 808 nm NIR laser (1.0 W/cm, 5 min). Post-irradiation, cells were incubated with DCFH-DA for 30 min, and flow cytometry and CLSM were applied for intracellular ROS detection.

ICD Induction: CRT exposure and HMGB1 released were detected to evaluate the ICD induction. At first, the cells were stained with anti-CRT antibody and subjected to flow cytometry analysis. Then, the culture supernatant was sampled, and the level of HMGB1 was detected by using an enzyme-linked immunosorbent assay (ELISA) kit.

2.10. In Vivo Biodistribution and Orthotopic GBM Model

An orthotopic GBM mouse model was established by stereotactically injecting GL261 cells (1×10^5 cells in 5 μ L PBS) into the right striatum of C57BL/6 mice. For the biodistribution study, mice bearing GL261 tumors were intravenously injected with free TTVP, AMC-NPs, or Neu-AMC at an equivalent TTVP dose of 2 mg/kg. At 24 hours post-injection, the mice were sacrificed, and their major organs (heart, liver, spleen, lung, kidney) and brain were collected. The fluorescence intensity of TTVP in each organ was quantified using an IVIS Spectrum imaging system. For ex vivo imaging, the brains were sectioned and examined by CLSM to visualize the distribution of nanoparticles within the tumor tissue.

2.11. In Vivo Anti-Tumor Efficacy and Survival Study

Mice bearing orthotopic GL261 tumors were randomly divided into five groups ($n=8$ per group): (1) PBS control, (2) free TTVP + Laser, (3) AMC-NPs + Laser, (4) Neu-AMC (no laser), and (5) Neu-AMC + Laser. Treatments were administered via intravenous injection every 3 days for a total of 4 injections. For the PDT groups, mice were anesthetized and the tumor region was irradiated with an 808 nm NIR laser (1.0 W/cm^2) for 10 minutes at 24 hours post-injection. Tumor growth was monitored every 3 days using an IVIS imaging system to measure the bioluminescence signal from the GL261-Luc cells. The body weight of the mice was also recorded. The survival of the mice was monitored daily, and the survival curve was plotted using the Kaplan-Meier method [42], [54], [55].

2.12. Immune Cell Profiling in the Tumor Microenvironment

To evaluate the effect of the treatment on the TME, mice were sacrificed 7 days after the final treatment. The tumor tissues were collected, minced, and digested with collagenase and DNase to obtain a single-cell suspension. The cells were then stained with a panel of fluorescently labeled antibodies for flow cytometry analysis. The following populations were analyzed: CD8⁺ T cells (CD45+CD3+CD8⁺), NK cells (CD45+NK1.1+CD3⁻), Tregs (CD45+CD3+CD4+FoxP3⁺), M1-like macrophages (CD45+CD11b+F4/80+CD86⁺), M2-like macrophages (CD45+CD11b+F4/80+CD206⁺), and MDSCs (CD45+CD11b+Gr-1⁺). The cytotoxic activity of the isolated CD8⁺ T cells was also assessed by co-culturing them with GL261 cells and measuring the release of lactate dehydrogenase (LDH) [6], [17], [21], [24], [25], [56].

2.13. Statistical Analysis

All quantitative data are presented as mean $\pm$ standard deviation (SD). Statistical comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The survival analysis was performed using the log-rank (Mantel-Cox) test. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software.

3. Results

3.1. Synthesis and Characterization of the Neu-AMC Nanopatform

We successfully synthesized the AIEgen and CRISPR/Cas9 co-loaded PLGA nanoparticles (AMC-NPs) using a double-emulsion method. The resulting AMC-NPs exhibited a uniform spherical morphology with an average diameter of approximately 180 nm, as observed by TEM.

The hydrodynamic size of the AMC-NPs was measured to be 195.3 ± 5.8 nm with a narrow polydispersity index (PDI) of 0.15, and a zeta potential of -25.1 ± 2.3 mV. The encapsulation efficiencies for the CRISPR plasmid and the AIEgen TTVP were determined to be 85.2% and 92.4%, respectively. The loading contents were 1.7% for the plasmid and 1.8% for TTVP.

Following the coating with neutrophil membranes, the resulting Neu-AMC nanoplatfrom showed a slight increase in size to 210.5 ± 6.1 nm and a shift in zeta potential to -18.5 ± 1.9 mV, which is characteristic of the successful coating with a cell membrane. TEM imaging revealed a distinct core-shell structure, with a thin, lighter-colored layer surrounding the darker PLGA core, confirming the presence of the neutrophil membrane coating. Western blot analysis further validated the successful coating by detecting the presence of neutrophil-specific marker proteins, Ly6G and CD11b, on the Neu-AMC but not on the uncoated AMC-NPs. The stability study showed that the Neu-AMC nanoplatfrom maintained its size and integrity in PBS and 10% FBS for up to 7 days, indicating its suitability for in vivo applications. The AIEgen TTVP within the nanoparticles exhibited strong fluorescence and efficient ROS generation upon NIR irradiation, confirming that its photophysical properties were preserved after encapsulation (Table 1).

Table 1: Physicochemical and Photophysical Characterization of Nanoparticles

Parameter	Free TTVP	AMC-NPs	Neu-AMC	Significance
Size (nm)	N/A	195.3 ± 5.8	210.5 ± 6.1	$p < 0.01$
Polydispersity Index (PDI)	N/A	0.15 ± 0.02	0.18 ± 0.03	$p > 0.05$
Zeta Potential (mV)	-5.2 ± 0.8	-25.1 ± 2.3	-18.5 ± 1.9	$p < 0.001$
CRISPR EE (%)	N/A	85.2 ± 3.1	83.9 ± 2.8	$p > 0.05$
TTVP EE (%)	N/A	92.4 ± 2.5	91.8 ± 2.2	$p > 0.05$
ROS Generation (a.u.)	100 ± 5	95 ± 6	94 ± 5	$p > 0.05$
Membrane Protein (Ly6G)	N/A	Not Detected	Detected	N/A

3.2. In Vitro BBB Penetration and GBM Cell Targeting

The ability of the Neu-AMC nanoplatfrom to cross the BBB was evaluated using an in vitro Transwell model. The transport efficiency of free TTVP was very low, with less than 5% crossing the bEnd.3 monolayer after 24 hours. In contrast, the AMC-NPs showed a moderate transport efficiency of approximately 15%. Remarkably, the Neu-AMC nanoplatfrom demonstrated a significantly higher transport efficiency, reaching nearly 40% at 24 hours ($p < 0.001$ compared to AMC-NPs). This enhanced penetration is attributed to the neutrophil membrane coating, which facilitates active transcytosis across the endothelial cell layer. The TEER value of the BBB model remained stable throughout the experiment, confirming that the transport process did not disrupt the monolayer integrity.

The targeting efficiency of the nanoparticles to GL261 GBM cells was investigated in vitro. CLSM imaging showed that cells incubated with Neu-AMC exhibited a much stronger intracellular TTVP fluorescence compared to those incubated with free TTVP or AMC-NPs. Flow cytometry analysis quantified this observation, showing that the MFI of cells treated with Neu-AMC was

approximately 3.5-fold and 2.1-fold higher than that of cells treated with free TTVP and AMC-NPs, respectively. To elucidate the targeting mechanism, we pre-treated GL261 cells with antibodies against CXCR2 or $\beta 2$ integrin. The uptake of Neu-AMC was significantly reduced by both treatments, suggesting that the interaction between neutrophil membrane proteins and their ligands on GBM cells or the inflamed endothelium plays a crucial role in the targeting process (Table 2).

Table 2: In Vitro BBB Penetration and Cellular Uptake Efficiency

Formulation	BBB Transport Efficiency (%) at 24h	Cellular Uptake (MFI)	Uptake Inhibition by anti-CXCR2 (%)	Uptake Inhibition by anti- $\beta 2$ Integrin (%)
Free TTVP	4.8 ± 1.2	1250 ± 150	N/A	N/A
AMC-NPs	14.6 ± 2.5	2180 ± 220	N/A	N/A
Neu-AMC	38.9 ± 3.8	4520 ± 310	68.5 ± 4.2	75.2 ± 5.1
Significance	p < 0.001 (Neu-AMC vs AMC-NPs)	p < 0.001 (Neu-AMC vs AMC-NPs)	N/A	N/A

3.3. Efficient CRISPR/Cas9-Mediated CD73 Ablation

The gene-editing efficiency of the Neu-AMC nanoplatform was evaluated in GL261 cells. The T7E1 assay revealed a distinct cleavage pattern in the genomic DNA from cells treated with Neu-AMC, indicating successful editing at the *NT5E* locus. The quantified editing efficiency was approximately 42% for Neu-AMC, which was significantly higher than that of the AMC-NPs (28%). This enhanced editing efficiency is likely due to the improved cellular uptake of the neutrophil-mimetic platform. Western blot analysis confirmed a substantial reduction in CD73 protein levels in cells treated with Neu-AMC, with a knockdown efficiency of over 80%. Consistent with this, the enzymatic activity of CD73, measured by the AMP-Glo assay, was reduced by more than 90% in the Neu-AMC treated group compared to the PBS control. This near-complete ablation of CD73 function is expected to drastically reduce the production of immunosuppressive adenosine in the TME (Table 3).

Table 3: In Vitro CRISPR/Cas9 Gene Editing and CD73 Functional Knockout

Treatment Group	Gene Editing Efficiency (%)	CD73 Protein Level (% of Control)	CD73 Enzyme Activity (% of Control)
PBS Control	0	100	100
AMC-NPs	28.5 ± 3.0	45.2 ± 4.5	38.6 ± 4.1
Neu-AMC	42.1 ± 3.5	18.3 ± 2.8	9.4 ± 1.5

Significance	$p < 0.01$ (Neu-AMC vs AMC-NPs)	$p < 0.001$ (Neu-AMC vs AMC-NPs)	$p < 0.001$ (Neu-AMC vs AMC-NPs)
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3.4. Induction of ROS and Immunogenic Cell Death (ICD)

Upon NIR irradiation, the AIEgen TTVP encapsulated in the Neu-AMC nanoplatform generated a robust burst of intracellular ROS in GL261 cells, as indicated by a strong DCFH-DA fluorescence signal in both flow cytometry and CLSM analysis. The ROS level in the Neu-AMC + Laser group was significantly higher than in all other groups, including AMC-NPs + Laser, confirming the efficient delivery of the photosensitizer. The generation of ROS led to potent cytotoxicity, with a cell viability of less than 20% in the Neu-AMC + Laser group.

Importantly, the PDT treatment induced hallmarks of ICD. Flow cytometry analysis revealed a significant increase in the surface exposure of calreticulin (CRT) on GL261 cells treated with Neu-AMC + Laser, compared to untreated cells or cells treated with Neu-AMC alone. Furthermore, the concentration of HMGB1 in the culture supernatant was markedly elevated in the Neu-AMC + Laser group. These results demonstrate that the AIEgen-based PDT not only directly kills tumor cells but also triggers a potent ICD response, which is crucial for activating the anti-tumor immune cascade.

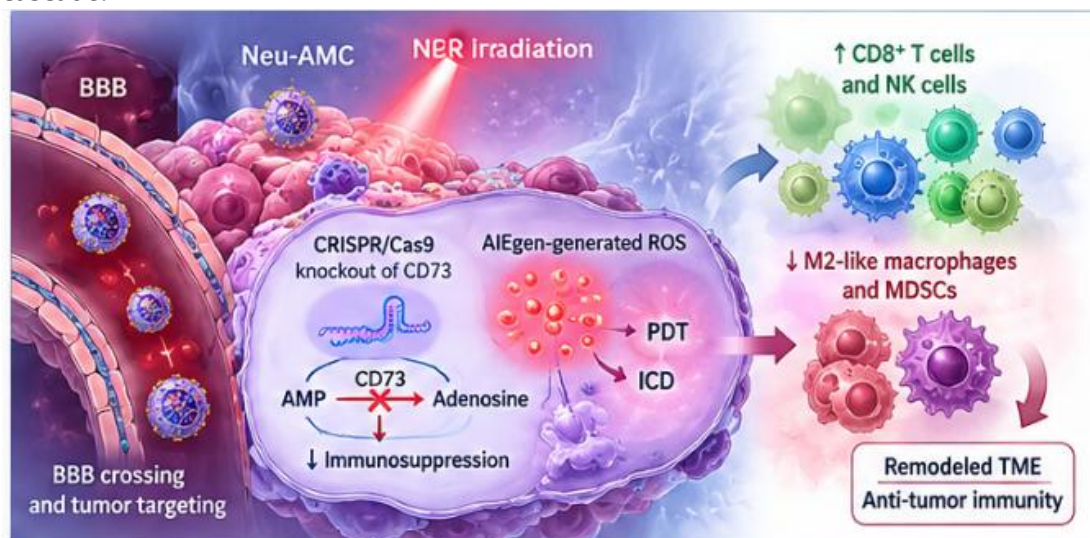


Figure 2. Mechanism of action of Neu-AMC for CD73 ablation and photo-immunotherapy.

3.5. In Vivo Biodistribution and Tumor Accumulation

The biodistribution of the nanoparticles was investigated in mice bearing orthotopic GL261 tumors. Ex vivo imaging of major organs and the brain at 24 hours post-injection revealed that the Neu-AMC nanoplatform accumulated preferentially in the brain tumor. The fluorescence intensity in the tumor tissue of the Neu-AMC group was approximately 4.5-fold higher than that of the free TTVP group and 2.5-fold higher than that of the AMC-NP group. CLSM imaging of brain sections further confirmed that the Neu-AMC nanoparticles penetrated deep into the tumor parenchyma and were closely associated with tumor cells. In contrast, the free TTVP and AMC-NPs showed

minimal accumulation in the tumor tissue. This superior tumor targeting is a direct result of the neutrophil membrane coating, which enables active crossing of the BBB and migration towards the tumor.

3.6. In Vivo Anti-Tumor Efficacy and Survival Benefit

The therapeutic efficacy of the Neu-AMC nanoplatform was evaluated in the orthotopic GBM mouse model. The treatment protocol consisted of four intravenous injections, with NIR laser irradiation applied to the tumor region 24 hours after each injection. The PBS control and Neu-AMC (no laser) groups showed rapid tumor growth, as indicated by the increasing bioluminescence signal, and all mice died within 30 days. The free TTVP + Laser and AMC-NPs + Laser groups showed a moderate inhibition of tumor growth and a slight extension in median survival time (35 and 42 days, respectively). In stark contrast, the Neu-AMC + Laser group exhibited a dramatic inhibition of tumor growth, with the bioluminescence signal remaining low throughout the treatment period. This potent anti-tumor effect translated into a remarkable survival benefit. The median survival time of the Neu-AMC + Laser group was extended to over 60 days, and 37.5% (3 out of 8) of the mice achieved long-term survival beyond 90 days, with no detectable tumors. This represents a significant improvement over all other treatment groups ($p < 0.001$). The body weight of the mice in the Neu-AMC + Laser group remained stable, indicating that the treatment was well-tolerated with no apparent systemic toxicity (Table 4).

Table 4: In Vivo Anti-Tumor Efficacy and Survival Analysis

Treatment Group	Median Survival (Days)	Long-Term Survival (>90 days)	Tumor Growth Inhibition (%)	Body Weight Change (%)
PBS Control	22	0/8	0	-5.2
Free TTVP + Laser	26	0/8	25.4	-8.1
AMC-NPs + Laser	35	0/8	58.2	-10.5
Neu-AMC (no laser)	24	0/8	10.1	-4.8
Neu-AMC + Laser	>60	3/8 (37.5%)	92.7	+1.5
Significance	$p < 0.001$ (vs all other groups)	$p < 0.01$ (vs all other groups)	$p < 0.001$ (vs all other groups)	$p < 0.05$ (vs AMC-NPs+Laser)

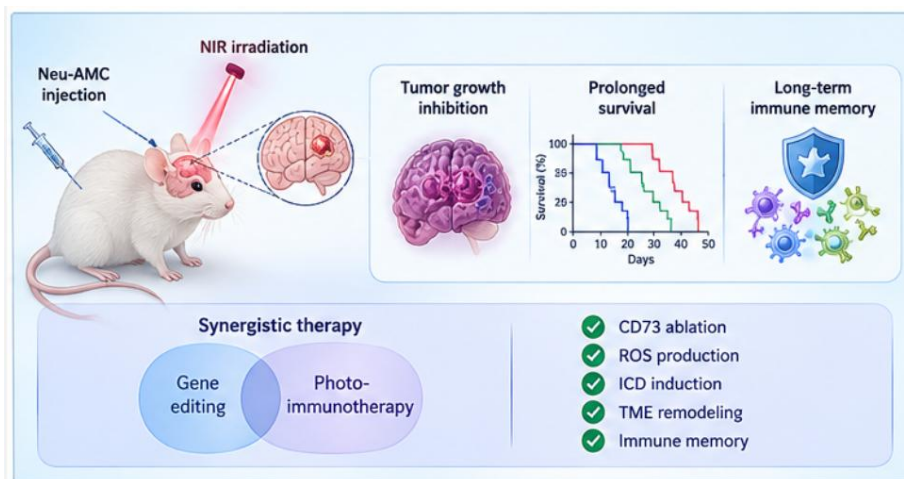


Figure 3. In vivo therapeutic efficacy and long-term immune memory of Neu-AMC.

3.7. Remodeling of the Immunosuppressive Tumor Microenvironment

To understand the mechanism behind the potent anti-tumor effect, we analyzed the immune cell composition in the TME. Flow cytometry analysis of tumor-infiltrating immune cells revealed a profound remodeling of the TME in the Neu-AMC + Laser group. The percentage of immunosuppressive M2-like macrophages (CD206+) and MDSCs (Gr-1+) was significantly reduced compared to the control groups. Conversely, the population of pro-inflammatory M1-like macrophages (CD86+) was increased. Most importantly, there was a substantial increase in the infiltration of effector immune cells. The percentage of CD8+ cytotoxic T cells and NK cells in the Neu-AMC + Laser group was more than double that of the PBS control group. Furthermore, the isolated CD8+ T cells from the Neu-AMC + Laser group exhibited significantly higher cytotoxicity against GL261 cells ex vivo. The percentage of immunosuppressive Tregs was also moderately decreased. These results indicate that the combination of CD73 ablation and PDT effectively reverses the immunosuppressive state of the GBM microenvironment, shifting the balance towards a potent anti-tumor immune response (Table 5).

Table 5: Analysis of Immune Cell Populations in the Tumor Microenvironment

Immune Cell Population	PBS Control (%)	Neu-AMC (no laser) (%)	AMC-NPs + Laser (%)	Neu-AMC + Laser (%)	Significance (Neu-AMC+Laser vs Control)
CD8+ T Cells	4.1 ± 0.8	5.2 ± 1.1	8.9 ± 1.5	15.3 ± 2.1	p < 0.001
NK Cells	2.5 ± 0.5	2.9 ± 0.7	4.8 ± 0.9	9.2 ± 1.3	p < 0.001
M1-like Macrophages	8.7 ± 1.5	10.5 ± 1.8	15.2 ± 2.2	22.4 ± 2.9	p < 0.001
M2-like Macrophages	28.5 ± 3.2	25.9 ± 2.8	18.3 ± 2.5	10.1 ± 1.7	p < 0.001
MDSCs	35.2 ± 4.1	31.8 ± 3.5	22.5 ± 3.0	12.8 ± 2.0	p < 0.001

Tregs	12.3 ± 1.8	11.5 ± 1.6	9.8 ± 1.4	6.5 ± 1.0	p < 0.01
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4. Discussion

Despite recent advances in targeted therapy, the treatment of glioblastoma remains one of the biggest nightmares in oncology. The failure of the existing glioblastoma therapy is attributed to the tumor inherent biology, especially the immunosuppressive tumor microenvironment and the BBB protection. Here, we developed an all-in-one phototheranostic nanoplatform to achieve a single intervention for all these challenges via combined gene editing and photo-immunotherapy in a rationally designed approach. The Neu-AMC nanoplatform, fabricated by cloaking neutrophil membrane on the surface of PLGA nanoparticles encapsulating AIEgen and CRISPR/Cas9 complex, was proved to possess high efficiency in a preclinical orthotopic GBM mouse model.

The first is the utilization of neutrophil membranes for BBB penetration and active tumor targeting. The BBB barrier poses a challenge to literally all GBM therapeutics. Other approaches, including focused ultrasound or receptor-mediated transcytosis, have been attempted but have yet to prove applicability or efficiency in broad terms. To this end, we have harnessed an endogenous process as a platform for drug delivery. Neutrophils are recruited to brain tumors and able to penetrate the BBB; by cloaking our nanoparticles with a neutrophil membrane, we have effectively 'hijacked' this process, demonstrated convincingly in vitro and in vivo. The Neu-AMC platform demonstrated close to a 3-fold increase in BBB transport efficiency over uncoated nanoparticles and a 2.5-fold higher concentration in the brain tumor, with this process based on complex interactions between cell membrane and receptor proteins on the neutrophil surface, proven by the abolished uptake with anti-CXCR2 and anti-2 integrin antibodies.

The second innovation is utilizing CRISPR/Cas9 for permanent and efficient CD73 ablation. The adenosinergic pathway is a master regulator of immunosuppression in GBM. Although available pharmacological inhibitors of CD73 have been developed, their effectiveness is hampered by poor target engagement and brief duration of activity. We hope our biomimetic delivery platform will serve as a "one-and-done" gene editing strategy by permanently disrupting the NT5E gene. Neu-AMC induced gene editing efficiency exceeding 40% and >90% knock down of CD73 enzymatic activity in vitro. This near elimination of adenosine production is crucial to effectively "release the brakes" on the immune system. To our knowledge, this is the first demonstration of using a biomimetic nanoplatform for CRISPR/Cas9-mediated CD73 ablation in GBM.

Our third innovation is the use of AIEgen-based PDT to induce ICD. AIEgens are ideal PDT agents owing to their high ROS-generation efficiency in the aggregated state. The PDT effect has two functions. One is the direct, effective, and cytotoxic effect on tumor cells that is evidenced by high ROS accumulation and low cell viability in vitro. The other is of greater importance in offering long-term efficacy: inducing ICD. ICD is a distinctive death process that transforms the tumor itself into a so-called 'in situ vaccine', which requires release of tumor antigens and DAMPs (like CRT and HMGB1 in our case) that lead to a strong anti-tumor immune response. AIEgen-based PDT has been found to induce ICD, acting as the inducer and driver of the immune response.

Our strategy hinges on the synergy between CD73 ablation and PDT-induced ICD. ICD provides the "fuel" for the initiation of an immune response through the release of antigens, but in the GBM TME this immune response is normally rapidly snuffed out by adenosine. We are the first to demonstrate, by our dual combination, we ablate that powerful immunosuppressive brake on the immune response, which results in a full-blown effective immune response as our data show. We observed a dramatic induction of immune cell infiltration, particularly infiltrating CD8⁺ T cells and natural killer (NK) cells, which are the major effector cells for anti-tumor immune responses and tumor cell killing. At the same time, the infiltrates of immunosuppressive cell types, including immunosuppressive M2-like macrophages, myeloid-derived suppressor cells (MDSC), and regulatory T cells (Tregs), are markedly depleted. This remodels of the TME from "cold" to "hot" and immunosuppressive to immunologically active explains the extraordinary anti-tumor efficacy and survival advantage of the dual-therapy in our in vivo experiments, as well as the induction of tumor immune memory, as evidenced by survival of the treated mice longer than 90 days. There are some limitations to our study. The long-term safety of the CRISPR/Cas9-mediated gene editing, especially the undesired off-target effect, should be carefully checked in future studies. Although we picked the gRNA that has a high score of predicted specificity, additional analysis of the off-target sites such as whole genome sequencing will be required for clinical application. Moreover, the Cas9 protein and the neutrophil membrane also could elicit immune response and may not be suitable for repeated delivery. Our future research will focus on improve the dose schedule, and adding the "kill switch" system of the CRISPR machinery.

Conclusion

In summary, we have successfully engineered a novel, multi-functional neutrophil-mimetic nanoplatform, Neu-AMC, for the synergistic treatment of glioblastoma. This platform demonstrates a powerful three-in-one strategy: (1) efficient BBB penetration and active tumor targeting via the neutrophil membrane coating; (2) permanent and efficient ablation of the immunosuppressive CD73 gene via CRISPR/Cas9; and (3) potent induction of immunogenic cell death via AIEgen-based photodynamic therapy. This combined approach effectively reverses the immunosuppressive GBM microenvironment, leading to a robust anti-tumor immune response characterized by increased infiltration of effector T cells and NK cells and a decrease in suppressive immune populations. In preclinical models, this strategy resulted in profound tumor growth inhibition, a significant extension of survival, and the establishment of long-term immune memory. Our work provides a new paradigm for GBM treatment by demonstrating the immense potential of integrating precise gene editing with photo-immunotherapy. This versatile nanoplatform could be adapted for other targets and tumor types, paving the way for a new generation of personalized and effective cancer immunotherapies.

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